Inhibition of Alopecia

The present invention relates to a process for inhibiting alopecia and a system of identifying alopecia-inhibiting substances.

Alopecia is a wide-spread hair disease which may result in the complete loss of the hair. The causes of alopecia are not known. In so far it is not possible to influence this disease in well-calculated fashion.

Therefore, it is the object of the present invention to provide a product by means of which this can be achieved.

According to the invention, this is achieved by the subject matters defined in the claims.

The present invention is based on the applicant's findings that certain forms of alopecia are based on an unbalanced keratinization of the hair. Furthermore, he has found that in the case of alopecia the mRNA of various genes is lacking, e.g. that of the Ha3 gene, or underrepresented, e.g. those of Ha1, Ha2, and Ha4 genes (cf. figs. 1 and 2). The gene products of Ha1, Ha2, Ha3, and Ha4 genes are hair keratins. The applicant has found that the expression of the Ha3 gene is controlled by a gene product of the whn gene. In particular, he has found that the expression of the Ha3 gene can be induced by the expression of the whn gene (cf. fig. 3). He has also found that the expression of other hair keratin genes is essentially influenced by the gene product of the whn gene. The applicant has also found that the expression of the whn gene varies in the course of the hair cycle. In particular, he has found that the whn expression in the telogen of the hair cycle drops to no longer detectable levels. In addition, he has discovered that the whn gene can be transcribed by two promoters. The applicant has obtained his findings by means of naked mice and HeLa cells.

According to the invention the applicant's findings are used for a process for inhibiting alopecia, which comprises the increase in the cellular amount of hair keratins.

The expression "increase in the cellular amount of hair keratins" refers to the fact that the amount of one or several hair keratins, particularly of Ha1, H2, Ha3 and Ha4, which may be present in small amount or not at all, is increased in cells. This can be achieved by common methods and substances, respectively. For example, one or several hair keratins, particularly Ha1, Ha2, Ha3 and Ha4, may be added to the cells as such or in the form of DNA encoding the same. The DNA may be present in common expression vectors. It is also possible to add substances which activate the expression of one or several hair keratins, particularly of Ha1, Ha2, Ha3 and Ha4. Such substances are e.g. the gene product of the whn gene or a DNA encoding the same. It may be present in common expression vectors. Moreover, substances may be added which activate the expression of the whn gene. They may also be present as such or in the form of DNA encoding the same, it being possible for the latter to be also present in common expression vectors. The expression "cells" comprises cells of any kind and origin. In addition, it comprises tissues and organisms, particularly animals and human beings.

Substances inhibiting alopecia can be administered as usual, preferably locally. The substances may also be present in common formulations. If the substances are administered locally, e.g. creams, ointments, shampoos and hair tonics will be suitable. The substances may also be present as particles which are easily absorbed. Examples of such particles are liposomes. A person skilled in the art knows processes to discover the suitable formulations and forms of administration, respectively, for the individual substances.

A further subject matter of the present invention relates to a system of identifying substances which are suited to inhibit alopecia. Such a system comprises the increase in

the cellular amount of hair keratins and/or substances activating the gene expression thereof. In particular, the system comprises animals or cells, cells being preferred, in which one or several expressible hair keratin genes and/or one or several expressible genes, whose gene products activate the gene expression of hair keratins, are present each in fused form with a reporter gene. The hair keratin genes may be particularly those of Ha1, Ha2, Ha3 and Ha4. Moreover, it is favorable for the substance activating the gene expression of hair keratins to be a gene product of the whn gene. In addition, the above genes may have a wild type sequence or a modified sequence, it being possible for the latter to differ from the wild type sequence by one or several base pairs. The differences may exist in the form of additions, deletions, substitutions and/or inversions of base pairs. Besides, an above reporter gene may be any gene, particularly it may code for an enzyme, e.g. alkaline phosphatase, or a fluorescent protein, e.g. GFP. The fusion genes may also be available in extrachromosomal fashion or in the cell genome, particularly in place of one or both allels of hair keratins and/or the genes whose gene products activate the expression of hair keratins. Besides, the system may contain substances which are suited to detect the expressed hair keratins and/or substances activating the gene expression thereof and the fusion genes, respectively. Such substances may be suited for the detection on a nucleic acid level and protein level, respectively.

By means of the present invention it is possible to inhibit alopecia. It is also possible to diagnose alopecia by determining e.g. the gene expression of hair keratins and/or substances which activate it. Moreover, it is possible to discover substances which are adapted to inhibit alopecia. For this purpose, a system is provided which is suited for the rapid and reliable screening of the most varying substances. Thus, the present invention provides products serving for diagnosing and treating a wide-spread hair disease.

Brief description of the drawings:

Fig. 1 shows an *in situ* RNA hybridization using a probe for mHa3 in normal (whn +/+) and mutant (whn -/-) mice. The transcripts for mHa3 (perceptible as brown silver grains) cannot be detected in hair follicles of naked mice. The line corresponds to 100 μm.

Fig. 2 shows the expression of whn and hair keratins in the hair follicle of a mouse.

A. Northern filter hybridization with RNA from the total skin of normal mice (whn +/+) and naked mice (whn -/-) by means of probes for hprt and whn genes as well as Ha1, Ha3, Ha4 genes at three times following the birth dP7, 7 days after the birth, etc.).

B. *In situ* RNA hybridization in the skin from normal (whn +/+) and naked mice (whn -/-) with probes for Ha1, Ha3, and Ha4 genes. An autoradiogram of skin cuts on day 7 after the birth is shown.

Fig. 3 shows the control of keratin gene expression. HeLa cells were transiently transfected with a whn expression construct (+), and the presence of Ha3-specific mRNA was detected by means of RT-PCR. The molecular weight markers are given in bp.

The present invention is explained by the below examples:

Example 1: Detection of the loss of expression of the Ha3 gene in mice suffering from alopecia

The "Representational Difference Analysis" (RDA) method was carried out. This method

comprises the isolation of mRNA from skin cells of (whn +/+) mice and (whn -/-) mice (mice suffering from alopecia and having no expression of the whn gene), respectively, the transcription of mRNA into cDNA, and the differentiation of the cDNA, thereby identifying the one underexpressed and overexpressed, respectively, in (whn -/-) mice.

A) Sequence of the oligonucleotide adapters

The following oligonucleotide adapter pairs were required for RDA:

R-Bgl-12: 5'-GATCTGCGGTGA-3'
R-Bgl-24: 5'-AGCACTCTCCAGCCTCTCACCGCA-3'

R-Bgl-12: 5'-GATCTGTTCATG-3'
R-Bgl-24: 5'-ACCGACGTCGACTATCCATGAACA-3'

R-Bgl-12: 5'-GATCTTCCCTCG-3'
R-Bgl-24: 5'-AGGCAACTGTGCTATCCGAGGGAA-3'

B) Production of poly A-mRNA from tissues to be compared with one another

First, RNA was obtained from the skin of (whn +/+) mice and (whn -/-) mice, respectively, according to the "single step RNA extraction" method (Chomczynski and Sacchi, 1987). The poly A-mRNA fractions from both RNA populations were then isolated by means of dynabeads oligo(dT) according to the corresponding protocol from the company of Dynal.

C) Synthesis of double-stranded cDNA

The "ribo clone cDNA synthesis kit" from the company of Promega was used for the synthesis of double-stranded (whn +/+) cDNA and (whn -/-) cDNA, respectively. 4 µg poly A-mRNA were used each to obtain about 2 µg cDNA.

D) Difference analysis

1. Restriction digestion of the double-stranded cDNAs

a) About 2 µg of each cDNA were digested in a 100 µl reaction batch by the restriction endonuclease DpnII at 37°C for 2 h.

b) The reaction solutions were then extracted twice using a phenol/chloroform mixture (1:1) and once using 100 % chloroform.

c) The DNA included in the aqueous phases of the two reaction batches was mixed with 2 µg glycogen, 50 µl 10 M ammonium acetate, and 650 µl 100 % ethanol each and precipitated on ice for 20 min.

Following 14 minutes of centrifugation at 4°C and 14,000 rpm, the supernatant was discarded and the DNA pellet was washed with 70 % ethanol. After another centrifugation and removal of the alcoholic phase, the dried DNA was resuspended in 20 µl TE buffer.

2. Ligation of the cDNAs to the R-Bgl oligonucleotide adapter pair

a) A reaction vessel collected the following:

20 µl cut cDNA (total reaction batch from item D)1c)
8 µg R-Bgl-24
4 µg R-Bgl-12
6 µl 10 x ligase buffer
x µl water
57 µl final volume

b) The reaction batch was heated in a thermocycler (Peltier Thermocycler PTC-200, MJ Research) to 50°C, kept at this temperature for 1 min., and then cooled again down to 10°C in the course of one hour (ramp rate: 0.1°C/9 sec).

c) After adding 3 µl T4 DNA ligase (1 U/µl), the mixture was incubated at 16°C overnight.

3. Synthesis of "representations" of the cDNA populations to be compared with one another

a) In order to generate what is called "representations" of the ligated cDNAs, the volume of the ligation batches from item 2c) was initially completed by adding 140 µl water each to give 200 µl.

Then, 30 reactions of 200 µl each were prepared from this dilute solution per cDNA population (whn +/+) skin and (whn -/-) skin.

The following reactants were added to such a batch one after the other:
143 µl water
20 µl 10x PCR buffer
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl R-Bgl-24 (1 µg/µl)
4 µl dilute ligation batch

b) PCR:
3 min.: 72°C
addition of 1 µl Taq-DNA polymerase (5 U/µl)
20 x: 5 min.: 95°C
3 min.: 72°C
finally: cooling to 4°C.

c) For preparing the reaction solutions, 4 reaction batches each were collected in a vessel.

Extraction: 2 x with 700 µl phenol/chloroform each (1:1), 1 x with chloroform 100 %;

Precipitation: addition of 75 µl 3 M Na acetate solution (pH 5.3) and 800 µl 2-propanol to each reaction vessel, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

Washing of the DNA pellets with ethanol 70 % and resuspension in such an amount of water that a concentration of 0.5 µg/µl resulted.

4. Restriction digestion of the "representations"

a) In order to remove the R-Bgl oligonucleotide adapters, 300 µg of each representation (whn +/+) skin and (whn -/-) skin, respectively, were subjected to restriction digestion. After adding the following reactants, incubation was carried out at 37°C for 4 hours:
600 µl cDNA representation (0.5 µg/µl)
140 µl 10 x DpnII buffer
100 µl DpnII (10 U/µl)
560 µl water.

b) The restriction digestion batch was distributed to 2 vessels prior to its preparation.

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %;

Precipitation: addition of 70 µl 3 M Na acetate (pH 5.3), 700 µl 2-propanol to each vessel, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.

The DNA pellet was washed with ethanol 70 % and resuspended in such an amount of water that a concentration of 0.5 µg/µl resulted.

The resulting DpnII-digested (whn +/+) skin cDNA representation represented the driver DNA population to be used for the subtractive hybridization.

5. Synthesis of the tester DNA population

a) 20 µg of the (whn -/-) skin cDNA representation digested by DpnII (=

tester DNA) was separated in a TAE gel by means of electrophoresis:
40 µl tester DNA (0.5 µg/µl)
50 µl Te buffer
10 µl 10 x loading buffer
were placed on a 1.2 % agarose TAE gel. A voltage was applied to the gel until the bromophenol blue component of the loading buffer had migrated about 2 cm.

b) Thereafter, the bands containing the representation DNA were cut out off the gel and eluted by means of the "agarose gel DNA extraction kit" from the company of Boehringer Mannheim.

The DNA extracts were collected, so that a total of 60 µl solution was obtained. The concentration of this solution was evaluated by electrophoresis of 5 µl in a 1 % agarose gel.

c) Eventually, the tester DNA was ligated with the J-oligonucleotide pair:
2 µg tester DNA eluate
6 µl 10 x ligase buffer
4 µl J-Bgl-24 (2 µg/µl)
4 µl J-Bgl-12 (1 µg/µl)
x µl water
57 µl final volume

d) Transferring the reaction batch to the thermocycler:
1 min.: 50°C
cooling down to 10°C within 1 h (ramp rate: 0.1°C/9 sec.)

e) The addition of 3 µl T4 DNA ligase (1 U/µl) was followed by incubation at 16°C overnight.

f) Adjustment of the concentration of the tester DNA to about 10 ng/µl by the addition of 120 µl water.

6. Subtractive hybridization

a) 80 µl driver DNA (40 µg) from step 4. and 40 µl (0.4 µg) dilute tester DNA from step 5., ligated with J-oligonucleotides, were collected in a reaction vessel and extracted 2 x with phenol/chloroform (1:1) and once with chloroform 100 %.

b) Precipitation by adding 30 µl 10 M ammonium acetate, 380 µl ethanol 100 %, -70°C for 10 min.

Centrifugation:	14 min., 14,000 rpm, 4°C
Thereafter:	2 x washing the pellet with ethanol 70 %, short centrifugation after each wash step; drying of the DNA pellet.

c) The DNA was resuspended in 4 µl EE x3 buffer (30 mM EPPS, pH 8.0 at 20°C (company of Sigma), 3 mM EDTA) - with pipetting off and on for about 2 min., then heated to 37°C for 5 min., shortly vortexed and eventually the solution was collected again at the vessel bottom

by centrifugation. Finally, the solution was coated with 35 µl of mineral oil.

d) Transferring the reaction batch to the thermocycler:

5 min.: 98°C,
cooling down to 67°C and immediate addition of 1 µl 5 M NaCl to the DNA,
incubation at 67°C for 20 h.

7. Synthesis of the first difference product

a) After removing the mineral oil as completely as possible, the DNA was diluted step-wise:
1. addition of 8 µl TE (+ 5 µg/µl yeast RNA),
2. addition of 25 µl TE - thereafter thorough mixing,
3. addition of 362 µl TE - vortex.

b) 4 PCRs were prepared for each subtractive hybridization. Per reaction:
127 µl water
20 µl 10 x buffer
20 µl 2 mM dNTPs
5 µl 25 mM Mg chloride
20 µl dilute hybridization solution (from step 7a))

c) PCR program:
3 min.: 72°C
addition of 1 µl Taq DNA polymerase (5 U/µl)
5 min.: 72°C
addition of 2 µl primer J-Bgl-24 (1 µg/µl)

10 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, then cooling down to room temperature.

d) The 4 reaction batches were collected in a 1.5 ml vessel.
Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
After the addition of 2 µg glycogen carrier:
Precipitation with 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.
Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70%.
After drying the DNA, resuspension in 40 µl water.

e) 20 µl of the resuspended DNA from d) were subjected to mung bean nuclease digestion (= MBN):
20 µl DNA
4 µl 10 x mung bean nuclease buffer (company of NEB)
14 µl water
2 µl mung bean nuclease (10 U/µl; company of NEB)
35 min., 30°C.

The reaction was stopped by adding 160 µl of 50 mM Tris-HCl (pH 8.9) and 5 minutes of incubation at 98°C. Thereafter, the vessel was placed on ice up to the next step.

f) During the MBN incubation, 4 further PCRs were prepared on ice:
127 µl water
20 µl 2 mM dNTPs
10 µl 25 mM Mg chloride
2 µl J-Bgl-24 (1 µg/µl)
20 µl MBN-digested DNA.

g) PCR program:
1 min.: 95°C
allowing to cool down to 80°C, addition of 1 µl Taq DNA polymerase (5 U/µl)
18 x: 1 min.: 95°C
3 min.: 70°C
finally: 10 min.: 72°C, allowing to cool down to 4°C.

h) The 4 PCR batches were collected in a vessel
Extraction: 2 x phenol/chloroform (1:1)
1 x chloroform 100 %.
Precipitation: 75 µl 3 M Na acetate (pH 5.3), 800 µl 2-propanol, 20 min. on ice.

Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the DNA pellet with ethanol 70 %.
Resuspension of the DNA in 100 µl water (resulting concentration: 0.5 µg/µl); the resulting solution represented the first difference product.

8. Exchange of the oligonucleotide adapters of the difference product

a) Removal of the oligonucleotide adapters by restriction digestion using DpnII:

40 µl difference product 1 (0.5 µg/µl)
30 µl 10 x DpnII buffer
15 µl DpnII (10 U/µl)
215 µl water
37°C for 2 h.

b) Preparation of the reaction batch:

Extraction: 2 x phenol/chloroform (1:1), 1 x chloroform 100 %.
Precipitation: 33 µl 3 M Na acetate (pH 5.3), 800 µl ethanol 100 %, -20°C for 20 min.
Centrifugation: 14 min., 14,000 rpm, 4°C.
Washing of the pellet in ethanol 70 % and resuspension in 40 µl water.

c) Ligation of the difference product to N-Bgl oligonucleotide adapter pair
1 µl of the prepared DNA solution from step b) was diluted with 9 µl water to give a concentration of 50 ng/µl; 4 µl of this solution were used in the following reaction:
4 µl DpnII-digested difference product 1 (200 ng)
6 µl 10 x ligase buffer
2.5 µl N-Bgl-24 (3.5 µg/µl)
2 µl N-Bgl-12 (2 µg/µl)
42.5 µl water.

d) After transferring the reaction batch to the thermocycler:
1 min.: 50°C,
allowing to cool down to 10°C within one hour (ramp rate: 0.1°C/9 sec.).

e) After adding 3 µl T4 DNA ligase (1 µg/µl), incubation at 16°C overnight.

9. Synthesis of the 2nd difference product

By adding 100 ml water, the ligation batch from step 8e) was diluted to a concentration of 1.25 ng/µl. 40 µl of this dilution (50 ng) were mixed with 80 µl driver DNA (see item 4.) and treated again according to steps 6. to 8. When the oligonucleotide adapters (step 8.) were exchanged, the J-Bgl oligonucleotides were then ligated to the newly formed difference product 2.

10. Synthesis of the 3rd difference product

The concentration of difference product 2 ligated with the J-Bgl oligos was reduced to a concentration of 1 ng/µl. 10 µl of this solution were diluted again with 990 µl water (+ 30 µg yeast RNA), so that the concentration was then 10 pg/µl. The subtractive hybridization was carried out with 100 pg (10 µl) J-ligated difference product 2 and 40 µg (80 µl) driver DNA from step 4.). As for the rest, the same steps were carried out as in the first and second difference products according to steps 6. to 8. An exception was the PCR following the MBN reaction (item 7.g) - here only 18 instead of 22 cycles were carried out.

11. Cloning of the 3rd difference product

The 3rd difference product was initially subjected to restriction digestion using DpnII so as to remove the oligonucleotide adapters. The reaction product was then applied to a TAE gel and separated by means of electrophoresis. The separated DNA bands were cut out off the gel, the DNA was eluted and cloned into a vector (pBS Not) cut by BamHI.

12. Characterization of the difference products

In order to confirm that the cloned DNA fragments were not method artifacts but sequences which were actually included in the investigated DNA representations, Southern analyses were carried out in which the investigated cDNA representations were hybridized with the radioactively labeled cloning products.

Thereafter, those DNA fragments which had proved to be "real" difference products in the Southern analysis were investigated by means of Northern hybridizations: RNAs were blotted from the investigated tissues ((whn +/+) skin cDNA and (whn -/-) skin cDNA) and hybridized with the radioactively labeled cloning products. By this, the differential expression of these sequences was confirmed in the investigated tissues. An analysis of the sequences led to the result that the Ha3 gene is not expressed in nu/nu mice (mice suffering from alopecia) (cf. fig. 1).

Example 2: Expression of hair keratin and whn genes in normal mice and mice suffering from alopecia.

RNA was isolated from the skin of differently old normal (whn +/+) and naked (whn -/-) mice, separated electrophoretically in agarose gels, transferred to filters and hybridized with gene-specific probes.

The employed probes were as follows:

mHa1: nucleotides 1331 - 1551; Gene Bank, accession No. M27734
mHa3: nucleotides 1007 - 1204; Gene Bank, accession No. X75650
mHa4: nucleotides 1303 - 1542, cf. Bertolino, A.P. et al., J. Invest. Dermatol. 94, (1990), 297 - 303
whn: nucleotides 1141 - 1374; Gene Bank, accession No. X81593

It showed that hair keratin genes and whn genes in mice suffering from alopecia are not expressed and expressed only slightly, respectively.

Example 3: Detection of the expression induction of the Ha3 gene by the gene product of the whn gene.

A whn gene tagged at the N-terminal epitope was inserted in the expression vector pTRE (Clontech). The resulting DNA construct was used for a transient transfection of the HeLa Tet-On cell line (Clontech) by means of the calcium phosphate coprecipitation method. The cells were treated with 5 µg/ml docycline directly afterwards. 1 mM sodium butyrate was

added 24 h later. The cells were harvested 48 h after the transfection and subjected to a RT-PCR method. The primers used in the PCR method were as follows:

hHa3:
5'-CTGATCACCAACGTGGAGTC-3',
5'-TACCCAAAGGTGTTGCAAGG-3'.
The PCR method included 35 - 40 cycles each of 30 sec. at 95°C, of 30 sec. at 58°C and of 1 min. at 72°C.

It showed that an expression of the Ha3 gene was induced by the expression of the whn gene. Parallel controls in which no transfection was effected by means of the whn gene, did not result in an induction of the Ha3 gene expression.

Example 4: Preparation of the system according to the invention

A BAC clone referred to as BAC whn, which comprises the entire whn gene of a mouse, was isolated from a BAC library of the company of Genome Systems (St. Louis, Missouri, U.S.A.) (cf. Schorpp, M. et al., Immunogenetics 46, (1997), 509-515).

In addition, a shuttle vector referred to as pMBO96-whn-GFP was used, which included the mouse whn gene which contained the reporter gene GFP in exon 3 (cf. Nehls, M. et al., Science 272, (1996),886-889).

BAC-whn was used to transform the recA$^+$ E. coli strain CBTS. The transformation was carried out by means of electroporation.

Clones were isolated and transformed by means of pMB096-whn-GFP using electroporation. A homologous recombination was made between the BAC clone and the shuttle vector within the range of the whn gene so as to obtain a vector referred to as BAC-whn-GFP. It included the reporter gene GFP in the whn gene.

BAC-whn-GFP was used for the transfection of COS cells. The transfection was carried out by means of the calcium phosphate coprecipitation method. COS cells were obtained which coded for a fusion gene from whn and GFP.

It showed that these cells were suited to identify substances which could induce the gene expression of whn. Such substances were suited to inhibit alopecia.